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METHODS OF STAINING NERVOUS TISSUE.

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DURING the past year a number of new staining methods have been discovered which enable the histologist to differentiate distinctly various parts of the nervous system. These methods have been published in different foreign journals, having originated for the most part in German laboratories. For the benefit of those who are studying nervous histology or pathology they are brought together and reproduced here, with a few additions which experience has suggested.

I.—WEIGERT'S HÆMATOXYLIN METHOD.

This is at present the most satisfactory of all the methods of staining, and is therefore given the first place. It brings out in strong contrast, even to the naked eye, the difference between gray masses and white fibres, and under the microscope differentiates so perfectly each fibre that it stands out like a stiff black hair in the field. The gray matter is colored a brownish yellow, the nerve cells being more deeply stained than the basis substance, and their nuclei and nucleoli coming out with great clearness. The network of fine fibres in the gray matter is demonstrated satisfactorily;

hence the method is of use in studying both the spinal cord, medulla, and brain cortex. Connective tissue does not retain the dye, and therefore in pathological specimens which contain sclerotic tissue this appears unstained and white in the dark field of normal substance. The sharp limitation visible to the naked eye makes class demonstrations of the location of system diseases easy, and under the microscope it is possible to contrast varying degrees of sclerosis, and even to count the number of axis-cylinders which have escaped destruction in the hardened area. The method is as follows: The specimen to be stained should be hardened in Müller's or Erlicki's fluid, and then cut into sections. If it has already been removed from the Müller's fluid and kept in alcohol it may be cut, but the sections must be placed in Müller's fluid for twenty-four hours before staining. In cutting the sections in either case the knife is to be wet with alcohol, and the sections are to be transferred to Müller's fluid and not to water or alcohol, as is customary. Having been placed in the Müller's fluid, and allowed to become thoroughly impregnated with it, the excess of fluid is removed by dipping the section for a moment into water, or by pouring a stream of water over it. The section must not, however, remain more than a minute in contact with water. It is then placed in the following solution of hæmatoxylin:

Hæmatoxylin (Merck's crystal.) . .	0.75
Alcohol (97%)	10.
Water	90.

This solution is prepared by boiling the hæmatoxylin in the alcohol and water until all the crystals are dissolved. It is then allowed to stand exposed to the light and air for four days before using. In placing the section in the hæmatoxylin it is well to spread it out flat upon a piece of tissue paper, as it becomes very brittle after absorbing the dye. The solution may be poured into a glass box, and the sections on their separate papers laid one upon another in it, but there should always be an excess of the dye. If the sections are large, the papers may be held apart by small bits of wood laid across their sides, in order to secure free access of the dye to all parts of the section, else the staining will be deeper at the edges than in the middle. Very large sections should be stained singly to prevent this.

The glass box containing the sections is then covered and placed in an oven, whose temperature is kept at a point between 35° and 45° C. (95°–112° Fahr.). It remains there an hour. If the temperature rises above 45° the process is not spoiled, but the specimens are liable to swell; if it remains below 35° the sections are not clearly stained. After being heated in this way the sections become very brittle, and therefore it is a good plan to immerse the box containing them in a large basin of water after pouring off the excess of dye. They can then be floated out of the box on the paper in the water, and washed by renewing the water in the basin. The washing should be continued until no more dye can be extracted from the section. It then appears uniformly black. It is then transferred to the following solution:

Ferricyanide of potash (red prussiate) . .	2.5
Borax	2.
Water	100.

in which it remains until a portion of the hæmatoxylin is removed. The length of time differs with different sections. They may be ready for removal in half an hour. They may have to remain for twenty-four hours.

The solution of potash has the power of dissolving the dye out of the gray matter and connective tissue rapidly, but acts very slowly upon that which has been taken up by the axis-cylinder and medullary sheath. The desirable degree of staining for the gray substance is a matter of personal taste. The longer the section remains in the potash solution the lighter will be the staining. For thin sections of the cord or medulla the time required in the potash is not more than an hour. The exact stage of decolorization can be watched if the specimens lie in white porcelain dishes. When they are sufficiently decolorized the sections are to be removed and washed in water until no more of the potash solution can be extracted. They may then be dehydrated by absolute alcohol, cleared up with oil of origanon, or cloves, or cedar, and mounted in Canada balsam. If a double stain is preferred they may be placed in a solution of alum-carmine before being dehydrated. This gives a pink color to the nerve-cells, but does not make them any more distinct than is done by the yellow potash. The only objection which can be made to this process is that the sections are rendered brittle by it, but if they are removed from solution to solution upon paper or a spatula, and not grasped at all by forceps, they need not be broken. Both the dye and the potash solution can be used a second time. It is sometimes impossible to conduct a staining process from beginning to end at one time. It may be a matter of convenience to leave the specimens indefinitely before the process is completed, They may be left in the staining mixture covered, or in the water after decolorizing with the potash, or in the alcohol used for dehydration, for any length of time. While they are in water after being dyed, and while they are in the potash solution, they should be watched. If left too long in the water they swell, if too long in the potash solution too much of the dye is removed.[1]

II.

Weigert has recently published[2] a modification of this method. He recommends that, after hardening in Müller's fluid, the specimen be placed in a saturated solution of neutral acetate of copper, which has been diluted with an equal part of water, and allowed to stand for two days in a heating box at a temperature of 40° C. From this it is removed to alcohol, in which it may be kept indefinitely. After cutting, the sections are placed directly in the hæmatoxylin solution, previously described, which is modified by the addition of a cold saturated solution of lithium carbonate in the amount of one part to one hundred of the dye. They remain in this for several hours, not being heated. Sections of spinal cord will stain in two hours; sections of brain must be left in a day. After removal they are treated as in the other method by the prussiate of potash. Weigert claims that the pigment produced by the union of the dye with the copper salt is more distinct and more sharply differentiated than that produced with the chromic salt. The addition of the lithium carbonate changes the color from black to dark blue. I find that sections which have been cut may be placed in the copper solution for a day and then stained by the aid of heat. If this is done, the differentiation in the prussiate-of-potash solution occurs much more rapidly, and must be carefully watched. The advantage of this modification is, however, not very great.

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[1] Weigert's original article is to be found in the *Fortschritte der Medicin*, Bd. ii., No. 6, 1884.

[2] *Fortschritte der Medicin*, April 15, 1885.

III.—WEIGERT'S ACID FUCHSIN METHOD.

This method, like the preceding, has for an object a sharp definition of nerve fibres. It accomplishes the object equally well with the hæmatoxylin method, but has certain disadvantages. The gray matter is less deeply stained than in the first method, and there is no contrast between the color of the gray and of the white, except one of shade, both being a magenta. The process is also a more complex one, though it has the advantage of being a rapid one.

The nerve fibres are well stained when the process succeeds, the nerve cells are slightly stained, the connective tissue is hardly colored at all. In sclerosis of the cord the method has much value, as the line of demarcation between normal and degenerated columns is distinct. In general myelitis it is not satisfactory, little differentation between the pathological products being obtained. It is evident, therefore, that nothing can be obtained by fuchsin which is not better obtained by hæmatoxylin. The method involves the preparation of the following solutions :

Sol. No. 1. A saturated watery solution of acid fuchsin (Fuchsin, S. No. 130 of the Baden anilin factory).

Sol. No. 2. A saturated absolute alcoholic solution of caustic potash, diluted with an equal part of alcohol.

Sol. No. 3. A five-per-cent. watery solution of hydrochloric acid.

The section, however hardened, is after cutting, to be first washed in water, then placed in Sol. No. 1, in which it remains one hour or longer, at the ordinary temperature. It is then removed and washed in water till the excess of the dye is taken off. It is then placed in Sol. No. 2, for a moment only, as it must be removed as soon as a distinction appears between the gray and the white matter. The gray matter will rapidly lose its color, while the section is in this solution, and the white matter will also be decolorized if the section is left in too long. A little experience will enable one to judge of the right moment for removal. The section is then placed in water, upon which it swims about as the alcohol evaporates, giving off red clouds of coloring matter. Here, too, it must be watched, and as soon as the clouds cease to be given off, it must be dipped in a fresh dish of water, and thence transferred to Sol. No. 3. This fixes the color definitely in the course of five minutes, when it is again washed in water, and then dehydrated with alcohol, cleared up with oil of cloves, and mounted in balsam.

If the section is thick it must remain several hours in the dye. If too much color is removed by Sol. No. 2, the section is to be washed in water and then replaced in the dye. If Sol. No. 2 has not taken out a sufficient amount of the color the section may be replaced in it at any time before it has been put in the oil of cloves. It is better, however, to have a specimen too deeply stained than too pale.

The sections do not become brittle in the dye, and no particular care is needed in the handling.[1]

Both of these methods of staining may be pursued with a specimen which has been embedded in celloidin. They have, therefore, some advantage over the carmine staining which has for so long been the favorite among neurologists. That staining is somewhat interfered with by the necessary soaking of the specimen in alcohol and ether, which precedes the embedding process. As no embedding mass can equal celloidin for convenience and cleanliness, this is an important consideration. It is to be remembered that celloidin is dissolved by oil of cloves. Oil of origanon may be used to clear the section which is embedded, as it does not dissolve the celloidin.

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[1] The original article of Weigert is to be found in the *Centralblatt für medicin. Wissensch.*, 1882, Nos. 40 and 42.

I have secured a pleasing result by combining these two methods; using first the hæmatoxylin and then the acid fuchsin. The gray matter is by this means stained red, and contrasts well with the black lines of fibres. The nerve cells become somewhat more distinct than by the yellow stain of the potash salts alone, and the network of fibres in the basis substance is very distinct. It has a little advantage, therefore, over the combination of hæmatoxylin with carmine suggested by Weigert.

IV.—SEGUIN'S MODIFICATION OF THE ANILIN BLUE METHOD.

Anilin blue-black, or nigrosene, was used as a staining color by Sankey as long ago as 1872. Its use was revived by Bevan Lewis in 1882. He found it especially advantageous as a dye for the cortex of the brain. The solution which he used was a dark, concentrated one, and after immersing the section in the dye it could not be seen, and consequently it was occasionally broken in being removed. On account of this objection Seguin tried a weak solution of a color not too deep to be translucent. He recommends a solution of one to three or even four thousand parts of water. The section is to be first soaked in water after being cut, as alcohol interferes with the staining process. It is allowed to remain in the solution of aniline blue for six to eighteen hours, when it becomes a dark blue color. It is then washed in water until a distinct difference is apparent between the gray and white matter, or until no more of the dye will wash out. The gray matter is seen to be a deep blue, and the white is a lighter shade. The specimen is then dehydrated with alcohol, cleared up by oil of cloves, and mounted in balsam.

This method stains the nerve cells a deep blue, but does not enable one to distinguish the nucleus from the cell body. The fibres are only lightly stained and do not come out with great distinctness. Connective tissue is stained deeply;

hence sclerotic tissue can be contrasted with normal tissue very well. The process is a simple one and easily carried out. The result is, however, not as satisfactory as the old carmine method.

If a section first stained in blue is then colored with acid fuchsin the appearance is very striking, as the mixture of the dyes makes the gray cells very distinct, and the fuchsin at the same time colors the fibres. Such a combination, however, involves considerable time, and the result, when attained, is in no respects superior to that reached by the simpler hæmatoxylin process.

V.—SAHLI'S DOUBLE STAIN WITH METHYL BLUE AND ACID FUCHSIN.[1]

The specimen must be hardened in Muller's fluid or in a 3–4 % solution of bichromate of potash. It is to be stuck upon a cork with gum arabic, and put in alcohol only long enough to fix the gum. Specimens preserved more than a few days in alcohol cannot be stained without being first soaked for an hour in the bichromate solution after being cut. If the specimen is embedded in celloidin it must be cut very thin, or the celloidin must be removed after cutting. After cutting the specimen the sections should not remain more than ten minutes in water. They are then placed in a concentrated watery solution of methyl blue for several hours, until deeply stained. They are then washed in water to remove superfluous dye, and placed in a saturated watery solution of acid fuchsin for five minutes. After the excess of fuchsin is removed by washing in water, the section is placed for a few seconds only in a 1 % alcoholic solution of caustic potash, and then again in water, where the differentiation takes place. The gray matter is stained red, the white matter is stained blue. Dehydration with alcohol, clearing up with oil of cedar, and mounting in balsam complete the process.

Sahli claims that when this stain does not succeed the difficulty lies rather in some defect in the hardening process than in a defect in the staining process. The fuchsin stains the axis-cylinder, the methyl blue stains the medullary sheath. In some fibres, however, the medullary sheath is stained with fuchsin instead of with blue, and Sahli hazards the conjecture that the difference of selective action of the sheath to absorb the blue or the red dye indicates a difference of function in the fibre which the sheath surrounds.

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[1] *Zeitschrift für Wissensch. Mikroskopie*, ii., S. 1.

Repeated trials of this process have convinced me that the varying colors obtained is a matter of accident, depending upon the length of time the section is left in the various fluids. If after staining with blue they are left too long in water much of the dye is dissolved out. If after staining with fuchsin they remain a few seconds too long in the potash too much of the fuchsin is dissolved out. Thus a slight variation in the process gives results which differ greatly. The conjecture of Sahli seems therefore unwarranted.

VI.—SAHLI'S METHYL BLUE STAIN.

Sahli has also proposed[1] a single stain obtained by placing sections, however hardened, for ten minutes in the following solution:

Sat. sol. of methyl blue	24 parts.
5 % sol. of borax	16 "
Water	40 "

Mix. Stand for twenty-four hours and filter.

They are then removed, washed in water to take off the excess of the dye, and dehydrated with alcohol. As the alcohol also dissolves out the dye they must not be left more than five minutes in it. They are then cleared up with oil of cedar and mounted.

This method has the advantage of being rapid and simple. It gives a clear blue color, and the fibres appear quite distinctly differentiated from the surrounding substance. The only objection is the rapid fading of the dye when exposed to light, which prevents its use for any specimen which is to be preserved.

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[1] In the same Journal.

VII.—THE SAFFRANIN STAIN.

This stain was proposed about a year ago by Adamkievicz,[1] and has been used by him in the study of pathological specimens. By means of it he claims to have observed a constant change in the vesicular column of Clarke in cases of locomotor ataxia, consisting in a disappearance of the fine reticulum of fibres which surrounds the cells of that column. The advantage of the stain is its power to bring out the finest fibres in the gray matter. The process is as follows:[2]

The specimen, however hardened, is cut, and the sections washed in water. They are then dipped in distilled water, which has been acidulated by a few drops of nitric acid. Thence they are transferred to a saturated solution of saffranin, where they remain from a few minutes to several hours, according to their thickness. Then the excess of the dye is removed by washing in alcohol, and the section is dehydrated by absolute alcohol. If a double stain is desired, it may now be washed again in water which has been acidulated with acetic acid, and then put in a concentrated solution of methyl blue. After a few hours it is taken out, washed in alcohol, dehydrated, and mounted. Specimens which have been hardened in alcohol show to better advantage by this method than those which have come in contact with chromic salts. The dye is taken up by the medullary sheath, and not by the axis-cylinder. The depth of color obtained will vary with the time the section remains in the dye, and the time it is allowed to stay in the alcohol subsequently to staining.

My own results with this method have not been satisfactory. So much of the dye is removed by the alcohol of dehydration that the specimen is very pale, and it is with difficulty that the fibres are seen. They certainly do not appear as distinctly as in the acid-fuchsin method.

These are the methods which, among a large number recently brought forward and tested, have seemed of sufficient value to warrant mention. Each has its peculiar advantages, as already stated. The hæmatoxylin and fuchsin methods of Weigert are of the greatest importance, and should be adopted by all who are doing pathological work here, as they have already been adopted in Germany. In staining embryonic tissue they have been of great service, and a number of discoveries of new tracts and bundles of fibres have been made by their aid. The dye in both cases is taken up by a portion of the medullary sheath, called the chromoleptic substance. Hence, in a fœtal brain, where some tracts are medullated and others non-medullated, these dyes select the medullated parts and define them clearly. In staining normal specimens they are also of service, since the various constituents of nerve tissue are differentiated distinctly. In staining sclerotic tissue their advantage has already been noticed. These methods are superior in all respects to the older carmine method, and should take its place.

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[1] *Anzeiger der K. Akad, d. Wissensch.*, iii. Abt., 1884, No. 7.

[2] For the details of this method, as well as for many useful suggestions regarding technique, I am indebted to Dr. C. F. Freeborn, Instructor of Technique at the Col. of Phys. and Surg., N. Y.

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